STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] II. THE EFFECT OF GAMMA RADIATION ON OXYGEN UPTAKE AND PHOSPHORYLATION

HENRY T. YOST, JR. AND HOPE H. ROBSON

Department of Biology, Amherst College, Amherst, Massachusetts

It is now well established that dilute solutions of many enzymes are readily inactivated by ionizing radiation and that the presence of solute molecules, other than enzyme, decreases the effect of the radiation (Barron, 1954). Since the classical experiments of Dale (1940, 1942), it has been possible to explain the effects of low dosages of radiation on the basis of interactions between the protein molecule and the ionization products of water. These findings have led to the discovery of important facts about radiation damage and about the nature of enzymes. However, an important question is raised by this work; that is, are we able to draw valid conclusions about the biological effects of radiation from such studies? It is evident that cellular enzymes are not in a pure state, nor are they as dilute as is necessary to achieve effects in some cases. This makes it necessary to investigate the effects of radiation on enzymes under conditions which approximate those of the cell.

There are two obvious ways to do this. The most usual method is to radiate a whole organism (or cell) and then determine the enzymatic activity after radiation. The results of such work indicate that the damage to enzymes by lethal doses of radiation may be negligible (LeMay, 1951). However, there are a number of obvious difficulties in such work; and since the organisms do die eventually and do show loss of respiration in some cases (Barron, 1954), one is left with an unsatisfied feeling. For this reason, it seemed advisable to study the effects of ionizing radiation on cellular particulates (Yost, Robson and Spiegelman, 1956). The particulates of intermediate size (mitochondria) offer several interesting possibilities for such investigations: they can be isolated from the cell in good condition; they contain a large number of vital enzymes; they have a definite structure to which some of the enzymes are attached; the enzyme studied would always be in an environment similar to, if not exactly the same as, that in which it finds itself within the cell; and most important, the particulate is sufficiently large that one might assume that a major part of the damage done to enzymes within the particulate would result from the passage of the ionizing "particle" through the mitochondrion itself. This would mean that the effects of the radiation on the enzyme studied would be the same whether the mitochondrion was extracted or within the cell.

The experiments reported in this paper had a two-fold purpose: the establishment of a dose-inactivation curve for an enzyme known to be closely associated with the structure of the particulate, and the determination of the effect of ionizing radiation on the phosphorylation mechanism. Cytochrome oxidase was chosen as the

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test enzyme since it is known to be closely bound to the particulate and therefore might be an indicator of effects of the radiation on the particulate as a whole, and since it is of vital importance to the electron transport system. Furthermore, the implication of the cytochrome system in the production of mutations by ionizing radiation (Haas *et al.*, 1954) suggests this enzyme as an excellent starting point for the investigation of the effects of radiations on cells.

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where much of the blood was washed free. The liver was weighed and pressed through a bronze screen to remove connective tissue. The resulting mash was then suspended in 50 ml. of cold 8.5% sucrose containing 0.005 *M* disodium versenate and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept.

In the studies of the effect of gamma radiation on the activity of cytochrome oxidase, the mitochondria were suspended in 2.5 ml. of sucrose-versenate per gram of original liver. For irradiation, a sample of the suspension was diluted 1 in 20 with distilled water. Five milliliters of the dilute preparation were put in a glass cup and irradiated in the beam of a 440-curie Co^{60} source. The radiation was filtered by a half-inch of lucite to remove beta radiation; the intensity of the radiation was 1000 r per minute. Controls were kept in a sheltered alcove outside the radiation room under the same conditions as the radiated material. The Warburg assays were run with the diluted preparations.

Treatment of the preparation for the determination of the effect of gamma radiation on phosphorylation differed from the above in some respects. The initial preparation was made by suspending the mitochondria in one ml. of sucrose-versenate per gram of liver. This suspension was then diluted 1 in 20 and radiated in a 25-ml. "Lusteroid" centrifuge tube. The controls were treated in the same manner, with the exception of the exposure to the radiation. After the radiation, control and treated suspensions were centrifuged, and the mitochondria re-suspended in one ml. This final suspension was assayed for phosphorylation.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml 1.3×10^{-4} *M* cytochrome-*c* (Sigma, horse-heart) in 0.85% NaCl, and 0.15 ml. 0.005 *M* $AlCl_3$. The center well contained 0.1 ml. 5 N KOH, and the side arm held the reducing agent, 0.15 ml. 0.228 *M* sodium ascorbate.

Estimation of phosphorylation was concluded by a modification of the method of Maley and Lardy (1954), using succinate as the substrate. The main compartment of the vessel contained: 0.3 ml. (30 μM) phosphate buffer (pH 7.4), 0.3 ml. 0.1 *M* sodium succinate, 0.8 ml. 8.5% sucrose, 0.1 ml. (0.3 μM) cytochrome-*c*, 0.3 ml. (6 μM) ATP (Schwartz, neutral), 0.1 ml. (30 μM) $MgSO_4$, 0.1 ml. (40 μM) KF, and 0.5 ml. of the mitochondrial suspension. The center well contained

0.1 ml. 5 N KOH, and the side arm held 0.5 ml. (20 mg.) of hexokinase (Pabst). To assure that the final pH of the reaction would be 7.0 or higher, the pH of some of the more acid reactants was adjusted with NaOH before addition to the flasks. Failure to do this results in lowered oxygen uptake and lowered phosphorylation. Readings of the oxygen uptake were taken for 30 minutes, after which time the reactions were stopped with TCA and the phosphate determined by the Lowry-Lopez method as presented by Glick (1949).

Assays of oxidase activity were made at 38° C.; assays of phosphorylation were made at 25° C. Assays of oxygen uptake were made in triplicate; assays of phosphorylation were made in duplicate. All experiments were repeated at least three times.

Results

Table I presents the data obtained from radiation of mitochondrial preparations of differing age. The preparation labeled "Day 1" was radiated on the same day

Table I

Inactivation of cytochrome oxidase by gamma radiation

Dose r	Day 1		Day 2		Day 3	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation	No. runs	Per cent inactivation
2,500	2	1.8±4.1	6	6.4±3.8	6	5.7±6.0
4,000	8	4.8±2.4	18	18.9±2.9	4	8.7±2.2
5,000	13	9.4±2.9	18	8.8±2.1	12	10.2±4.4
10,000	6	10.5±2.2	10	6.5±1.8	9	10.5±2.6
12,500	8	16.7±2.4	8	25.7±4.0	10	27.1±3.2
15,000	14	17.4±2.9	17	26.7±2.0	7	26.5±1.6
20,000	19	29.9±2.4	16	29.7±2.3	16	26.7±2.1
30,000	5	41.3±3.5	11	34.6±2.5	6	31.0±6.5
40,000	6	49.1±2.8	10	42.8±4.5	12	34.1±3.3

that it was extracted; the preparation labeled "Day 2" was radiated on the following day; etc. These data show that cytochrome oxidase is extremely resistant to gamma radiation. This is in accord with the earlier studies of Barron *et al.* (1949). Nevertheless, although the data are extremely erratic below 10,000 r, some effect is achieved with doses as low as 2500 r. There is little indication that a maximum has been reached at 40,000 r. It is necessary to comment on the variability shown by the data. The figures are averages of several runs done with different rats. We have found that preparations from different rats give different results. In the case of the data obtained with 4000 r, Day 2, the inactivation varied from 12.1 per cent to 36.5 per cent. Whether this intrinsic variability is the result of differences in age, sex, or physiological condition, we are unable to judge at this time. In addition, there is always the problem of variation in the concentration of the preparations. All dilutions are made from suspensions made up as 2.5 ml. per gram of liver extracted. There is no reason to suppose that the number of mitochondria will be the same in each case. Until studies are done in

TABLE II

The effect of aging on the inactivation of cytochrome oxidase

Dose r	Morning		Afternoon	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation
5,000	4	0.0±2.0	4	16.9±2.0
15,000	4	7.4±1.5	4	21.9±2.4
20,000	4	24.5±4.5	4	39.6±1.8

which the number of mitochondria in each sample are the same, no conclusions can be drawn about the variability between rats.

The data in Table I indicate that the preparations become more sensitive to low doses of radiation with time. On the first day, a dose of 2500 r produces little or no inactivation; on the second day, it produces about 6 per cent. This is shown in a much more striking manner in Table II. In this case the data from some runs done on the first day are broken down into those done in the morning and those done in the afternoon. In all cases, a preparation was radiated in the morning and afternoon, the only difference being the age. It is evident that when the mitochondria are first extracted they are much more resistant to radiation, particularly at the lower doses. At high doses, Table I indicates that there is a progressive decrease in sensitivity with age. Intermediate doses are erratic. As a result of

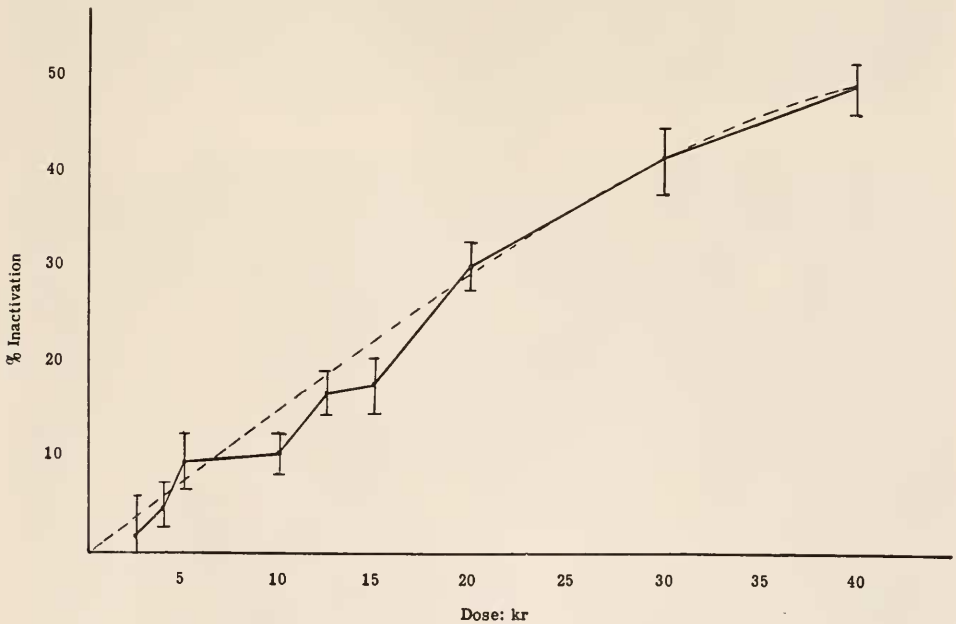

FIGURE 1. Inactivation of cytochrome oxidase by gamma radiation. Points represent means, with standard errors shown as limits. Preparation radiated the same day it was extracted. The dotted line represents the best fit of a regular curve.

this aging effect, the inactivation curve shown in Figure 1 was constructed on the basis of the first-day results only.

The data presented in Table III show that the oxidative phosphorylation mechanism is far more sensitive to radiation than cytochrome oxidase. All runs were done with a fresh preparation. There is no appreciable difference in the age of the preparations. In this case, each run represents a different rat, so that the variability results from this alone. It can be seen that there is little effect of the radiation upon the oxygen uptake in any case. The stimulation of phosphorylation by 2500 r is slightly greater than would be expected on the basis of increased

TABLE III

Inactivation of phosphorylation by gamma radiation

Dose	Phosphate uptake:		Per cent decrease	No. treated	O_2 uptake, % decrease
	Controls	Treated			
2,500	6.6±1.0	6.8±0.64	−3.0	10	−9.3
5,000	8.0±1.3	7.5±1.1	6.2	10	7.9
10,000	6.7±1.1	5.1±0.62	23.8	10	6.5
15,000	9.1±1.4	6.4±0.47	29.6	11	5.3
20,000	9.2±2.5	4.4±1.3	52.2	3	7.5
30,000	11.8±2.6	4.9±1.0	58.5	5	5.4
40,000	6.2±0.93	1.7±0.36	72.6	6	8.3

oxygen uptake, when calculations are made using a theoretical P:O ratio of 2 for succinate.

In all tables the data are shown as means ± standard error.

DISCUSSION

The data presented in this paper indicate that the inactivation of cytochrome oxidase by gamma radiation follows an irregular course. In all cases, there is a plateau reached at 5000 r to 10,000 r, followed by a sharp increase between 10,000 r and 12,500 r. The plateau is not lost upon aging (if anything, it is intensified); nor is it absent in fresh preparations. Table II indicates that in very fresh preparations there is a sharp increase between 15,000 and 20,000 r. It appears that the age of the preparation merely increases the dose necessary to cause the "jump." This observation requires special consideration. In the oxidation of cytochrome-*c* by ionizing radiation, it has been shown that the effects of radiation doses below 10,000 r are completely reversible (Barron, 1954). It is possible that part of the curve (Fig. 1) between 10,000 r and 15,000 r represents a shift from indirect to direct effects upon the particulate. Since we are radiating the entire particulate system, we can expect that the terminal oxidase of this system is constantly being reduced by substrate (either the supplied cytochrome-*c* or internal metabolites). This continual reduction may protect the cytochrome oxidase from the effects of the radiation, either during the time of radiation, when it must draw upon its internal supplies, or during the assay procedure, when it is supplied with a reducing agent which may effect post-irradiation recovery. Support for this idea comes from the aging effect shown in Table II. If the oxidase were being pro-

tected by materials within the mitochondrion at the time of radiation, we would expect that aging the preparation would lead to depletion of the internal stores with a consequent lowering of the protective effect. This is what is observed. At higher doses this protective effect is apparently negligible compared to the dose administered. Thus the effect of the radiation is reduced with age as might be expected from the protective effect of particulates whose oxidase has been inactivated by causes other than radiation.

It may be argued that the protection is merely the result of the various solute molecules which are not removed by washing during the extraction. It is difficult to decide whether there is a specific type of protection resulting from the generation of "reducing power" from the substrate, or whether there is a non-specific protective effect of additional substrate (Dale, 1942). It is clear that the effective substance is lost during aging; therefore, it seems sure that the protective substance must be within the particulate to be effective. If the substance were merely lost from the mitochondrion, it should be in the suspending medium which is diluted before radiation. The same concentration of substance would be present in either case. Such considerations raise the question of whether the effects of the radiation are independent of the concentration of the mitochondria. It might be suspected that only those ionizations produced within a mitochondrion would affect the enzymes within the structure. The particulate is sufficiently large to justify such an assumption. However, this does not prove to be the case. It was first established that preparations diluted in sucrose were less easily inactivated than those diluted in distilled water. This suggests that the ionization products of water are acting on the mitochondrion and that the sucrose is acting as a non-specific protective agent. Unfortunately this is not a clear test. The sucrose might be causing a change in the osmotic condition of the particulate, so that the protective substance (normally lost by aging) is more concentrated within the mitochondrion, or is not lost as readily. However, the data presented in Table III indicate that there is a dependance upon dilution. It can be seen that in a preparation 2.5 times as concentrated as that used to obtain the data in Table I, 40,000 r produce 8.3 per cent inactivation of oxygen uptake, whereas they produce 49.1 per cent in the dilute preparation. Clearly, 49.1 per cent is much greater than 2.5 times 8.3 per cent. Studies of intermediate dilutions bear this out. It is necessary to conclude that the effect of radiation upon the particulates is indirect in the sense that any solute molecule outside the mitochondrion will exert some protective effect upon the enzymes which are internal. Therefore, we must further conclude that the effect of the protective substance which is lost upon aging must be the result of some action it has *within* the mitochondrion, and that the loss which occurs with age probably results from the destruction of the substance by the particulates. As pointed out above, it is impossible to be sure of the mode of action of this protective agent, but the generation of materials which keep the enzymes in a reduced condition seems a likely mechanism.

A second explanation of the sudden "jump" in inactivation is that at doses over 10,000 r the particulate structure is undergoing severe change. This might result in the freeing of enzyme molecules, with consequent dilution of protective substances or in the loss of function of one part of the system with its release from another part. This seems a very unlikely mechanism. The fact that cytochrome oxidase can be freed from the rest of the particulate and still retain its activity

(Eichel *et al.* 1950) makes such an hypothesis difficult to maintain. Examination of the preparations by phase contrast did not show any radical changes; however, any alterations of ultrastructure could only be detected by other methods.

The dilution effect deserves additional comment. These experiments were originally designed in the hope that we would be able to approximate the biological condition. Radiation studies with dilute solutions of pure enzymes are informative with regard to radiation problems, but somewhat off the point for biological problems. No cellular system exists which is a single molecular species. Attempts to show that enzyme damage is the cause of radiation death in cells have been relatively unsuccessful (LeMay, 1951). On the other hand, the cell is so complex that it may die from many different causes, and it is difficult to be sure that one is investigating the right system in any particular case. It seemed necessary to investigate the problem in a system which had biological characteristics but which was not quite so diffuse as a whole cell. The particulates appear to offer such a system. It is possible to extract a "package" of enzymes, each of which has a relationship to other enzymes in the "package." The "package" resembles a cell in many aspects, but it is much simpler in its total organization. Furthermore it seemed that it was of sufficient size that only those ionizations produced within the mitochondrion would have any effect on the internal enzymes. This would be of great importance to the radiobiologist since it would indicate that there are sub-cellular bodies which can be considered to be separate from the rest of the cell, with regard to radiation damage. The chromosomes are frequently considered to be bodies of this type. Unfortunately this does not seem to be the case. The data presented in this paper indicate that ionizations external to the mitochondrion may cause internal damage, in a system containing only particulates and distilled water. It would appear that, at the doses studied, the ionization products of water are capable of producing their effects over relatively great distances, or that the effects of these products on the surface of the mitochondrion are capable of reducing the activity of the enzymes which are internal. This is of special interest as it has been suggested that the major effect of radiation on cytochrome-*c* is produced by hydroxyl radicals alone (Barron, 1954). Knowledge of the exact position of the oxidase in the particulate would be necessary to any final conclusion about these effects. However, the difficulties encountered in the extraction of cytochrome oxidase (Eichel *et al.,* 1950) suggest that the enzyme is internally bound.

From the foregoing discussion, it seems evident that we cannot conclude that cytochrome oxidase is damaged to any great extent by radiation doses used in most biological studies. It is difficult to know the concentration of the particulates in any cell, but it seems that the final suspension used in the phosphorylation studies (one ml. per one gm. liver) would best approximate the natural condition in liver cells. At no time was radiation given to particulates at this dilution. The strongest preparation ever used (during radiation) was 20 times diluted. At this dilution (Table III) there is little inactivation at 40,000 r. It is interesting that the effect on the oxidase seems to be the same for a wide range of doses. It is possible that in any dilution some small fraction of the activity would be lost, but this seems to be an unlikely cause of cell death. It is necessary to note that many cells do not have the high concentration of particulates which liver has. In these cases the effective doses necessary to inactivate cytochrome oxidase might fall within the

limits of biological experimentation, even with the protective substance of the cytoplasm present. In highly organized forms, the failure of one part may result in the death of the whole, so that damage to this system might *in some cases* result in death. This is particularly true of the forms which require fantastic doses of radiation to induce lethal changes.

When one considers the data in Table III, it becomes apparent that the oxygen uptake may be a faulty criterion for estimate of the health of a cell. These data show quite clearly that the phosphorylation mechanism is much more sensitive than the oxidase. Here we have a case in which over 70% of the ability to conserve energy as organic phosphate is gone with no apparent effect on the oxygen uptake. Considering the vital role of this system in the life of the cell, it seems quite probable that disruption of vital processes would result from the loss of 25 per cent (or less) of the ability to phosphorylate. We do not know how prevalent this loss is in the whole system. In these studies only the uptake which resulted from the oxidation of succinic acid was measured. It is possible that the whole phosphorylation mechanism of the particulates is damaged. Studies to determine the extent of the damage and to determine whether phosphorylation is carried out by a single system for all substrates are now in progress. In any case, it is evident that the phosphorylation mechanism is subject to destruction by ionizing radiation and that inactivation is achieved in relatively concentrated preparations which are very fresh. Although such preparations must be high in the concentration of the protective substance found for cytochrome oxidase, there seems to be little protection of the phosphorylation mechanism. This may be an indication that the protection mechanism is specific for the electron transport system (if not for cytochrome oxidase itself) or that the phosphorylation mechanism is extremely sensitive to radiation.

These data suggest an explanation for several different phenomena which have radiation as their sole common element. The phenomena are: induced crossing-over, induced tumor formation, and the general protective effect exerted by reducing compounds of the cysteine type. It has been suggested that one basis for the changes in genetic crossing-over induced by radiation is the alteration in the availability of phosphate linkages within the chromosome (Yost and Benneyan, 1957). It is evident that alterations in the phosphate pool of the cell must result from the type of damage described in this paper. Indeed, it is to be expected that such changes will have drastic effects on the chromosome structure, as many studies have already indicated (Haas *et al.*, 1954). It is also possible that *some* radiation-induced tumors are the result of the uncoupling of the oxidative metabolism of the cell. This could result in a situation similar to that which Warburg has suggested several times (Warburg, 1956). In cases in which oxygen uptake alone is measured, there is no assurance that the phosphorylation mechanism is functioning. Lastly, the general effect of reducing agents may be more than the maintenance of vital sulfhydryl groups. It is possible that the actual utilization of these compounds as reducing agents in the general metabolism will result in the protection of many non-sulfhydryl systems of the cell.

Summary

Data are presented which indicate that the phosphorylation mechanism is much more sensitive to gamma radiation than cytochrome oxidase. It is suggested that

the utilization of substrates by enzymes within the particulate may protect these enzymes against ionizing radiation. Various consequences of these findings are discussed.

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